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Reduction of Olive Knot Disease by a Bacteriocin from *Pseudomonas syringae* pv. ciccaronei†

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A bacteriocin produced by *Pseudomonas syringae* pv. ciccaronei, used at different purification levels and concentrations in culture and in planta, inhibited the multiplication of *P. syringae* subsp. *savastanoi*, the causal agent of olive knot disease, and affected the epiphytic survival of the pathogen on the leaves and twigs of treated olive plants. Treatments with bacteriocin from *P. syringae* pv. ciccaronei inhibited the formation of overgrowths on olive plants caused by *P. syringae* subsp. *savastanoi* strains PVBa229 and PVBa304 inoculated on V-shaped slits and on leaf scars at concentrations of 10^5 and 10^8 CFU ml^{-1}, respectively. In particular, the application of 6,000 arbitrary units (AU) of crude bacteriocin (dialyzed ammonium sulfate precipitate of culture supernatant) ml^{-1} at the inoculated V-shaped slits and leaf scars resulted in the formation of knots with weight values reduced by 81 and 51%, respectively, compared to the control, depending on the strains and inoculation method used. Crude bacteriocin (6,000 AU ml^{-1}) was also effective in controlling the multiplication of epiphytic populations of the pathogen. In particular, the bacterial populations recovered after 30 days were at least 350 and 20 times lower than the control populations on twigs and on leaves, respectively. These results suggest that bacteriocin from *P. syringae* pv. ciccaronei can be used effectively to control the survival of the causal agent of olive knot disease and to prevent its multiplication at inoculation sites.

Many plant pathogens produce bacteriocins, proteinaceous compounds which are active against closely related bacteria (4, 20). Among plant pathogenic pseudomonads, about 10 species or subspecies are reported to produce bacteriocins (6, 19). Syringacins 4-A and W-1 are high-molecular-weight and heat-sensitive bacteriocins identified and purified from two strains of *Pseudomonas syringae* pv. syringae isolated from corn and beans, respectively (5, 17). Applicative studies on disease control using bacteriocin-producing bacteria have also been reported (1, 13). In particular, Chen and Echandi (1) prevented the infection causing bacterial wilt of tobacco by dipping plants in a suspension of an avirulent bacteriocin-producing strain of *Ralstonia solanacearum*; they correlated the protection with bacteriocin production. Sakthivel and Mew (13) reported that treatment with nonpathogenic bacteriocin-producing strains of *Xanthomonas campestris* pv. oryzae reduces the incidence and severity of bacterial leaf streak in rice plants.

Olive knot disease, caused by *P. syringae* subsp. *savastanoi*, is characterized by hyperplasia formation on the stems and branches of olive plants and occasionally on the leaves and fruits (18). The disease can cause severe damage in olive groves, mainly when weather conditions favor the survival of epiphytic populations of the pathogen and their entry into the bark. *P. syringae* subsp. *savastanoi* has epiphytic resident populations on olive twigs, leaves, and drupes, with larger populations on twigs than on leaves as a result of the better survival of the pathogen in the rough bark surface (8). Population sizes were influenced by seasons, reaching about 10^4 bacteria/cm^2 (on twigs or leaves) in spring and fall, when wet weather conditions occur (3, 8). Tissues can be infected through leaf scars, wounds, and fissures on stems and twigs, caused by meteorological phenomena and insect miners, as well as by harvest and pruning practices. Schroth et al. (14, 15) reported that both olive yield and quality can be reduced as a consequence of bacterial infections of the plant by the pathogen. Actually, *P. syringae* subsp. *savastanoi* is included in the list of transmissible agents of olive diseases, and its absence in propagating material is advisable for the certification of olive mother plants in Italy (12).

Although control of olive knot disease is very difficult, the use of copper compounds may be one of the conventional practices to reduce symptoms. However, the diffuse resistance to copper bactericides among pathovars of *P. syringae* (21) requires the development of alternative control methods for bacterial pathogens, such as the use of biological control products to lower toxic pesticide residues on fruits and vegetables and to avoid environmental accumulation of chemicals and the consequent development of resistance among pathogens. Recently, Lavermicocca et al. (10) purified a bacteriocin produced by *P. syringae* pv. ciccaronei, a bacterium isolated from a leaf spot lesion on a carob tree. The bacteriocin selectively inhibited the growth of *P. syringae* subsp. *savastanoi* (10).

This investigation reports on the potential of bacteriocin preparations in controlling symptom expression of olive knot disease and in reducing the epiphytic survival of the pathogen on olive phylloplane.

MATERIALS AND METHODS

Bacterial strains. *P. syringae* pv. ciccaronei strain NCPPB2355 (National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom) and *P.*

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† Dedicated to Professor Antonio Graniti on the occasion of his 75th birthday.

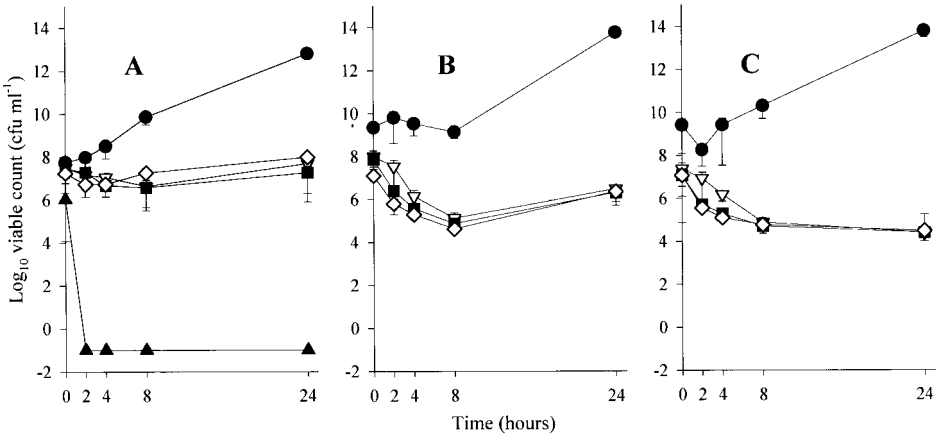

FIG. 1. Effects of *P. syringae* pv. ciccaronei NCPPB2355 bacteriocin preparations (A to C) and Kocide 101 solution (A) on the viability of growing cells of *P. syringae* subsp. *savastanoi* PVBa229. Symbols: ●, PVBa229; ▽, cs; ■, cb; ◇, fIV, ▲, Kocide 101 (k). The substances were added to final concentrations of 100 (A), 1,200 (B), and 6,000 (C) AU ml^{-1}. Kocide was added at a final concentration of 0.03%. Data, expressed as means ± standard errors (calculated at a confidence level of 95%), are from three independent experiments with two replicates each ($n = 6$).

syringae subsp. *savastanoi* strains PVBa229 and PVBa304 (collection of the Dipartimento di Patologia Vegetale, University of Bari, Bari, Italy) were stored and subcultured as previously reported (10). The *P. syringae* subsp.*savastanoi* strains used were randomly selected from 30 strains preliminarily tested with the bacteriocin (10).

Bacteriocin preparations and chemicals. *P. syringae* pv. ciccaronei NCPPB2355 was grown in Woolley's medium (23) supplemented with Bacto Peptone (Difco; 15 g $liter^{-1}$). The bacteriocin was prepared at different purification levels, i.e., culture supernatant (cs), crude bacteriocin (dialyzed ammonium sulfate precipitate; cb), and fraction IV (the most active fraction group recovered from Sephadex G-150 column; fIV), as previously described (10), and used for both in vitro and in planta experiments. Growth inhibition of the bacterial strains was expressed in arbitrary units (AU) of activity $milliliter^{-1}$ (10). The effect of bacteriocin was compared with the effect of the commercial bactericide Kocide 101 (cupric hydroxide; Griffin Corporation, London, England).

In vitro assays. The viability of cells of *P. syringae* subsp. *savastanoi* strains PVBa229 and PVBa304 exposed to bacteriocin preparations was examined. Strains were grown overnight with shaking in a modified Woolley's medium in which phosphates were used at a final concentration of 1 mmol $liter^{-1}$. One milliliter of the suspension (about 10^8 CFU ml^{-1}) was inoculated in each 25-ml Erlenmeyer flask containing 9 ml of fresh medium with 0, 100, 1,200, or 6,000 AU of the bacteriocin preparations ml^{-1} or with Kocide 101 (final concentration, 0.03% [wt/vol]). The number of viable bacterial cells was determined at different intervals on King's B medium (KB) (7) agar plates. Each assay was performed in duplicate in three separate experiments.

In planta experiments. For all experiments, 2-year-old olive plants (*Olea europaea* L. cv. Nocellara del Belice) were used. Plants were incubated in a growth chamber (Fitotron; Sanyo Gallenkamp, Leicester, England) (70% relative humidity, 15 h of light, 20,000 lux, 25°C; 9 h of dark, 22°C). Two methods of inoculation were employed. (i) For the first method, 10 μl of bacterial suspension containing 10^5 CFU of strain PVBa229 or PVBa304 ml^{-1} was spotted on three V-shaped slits (about 2 mm deep by 3 mm wide) made in the bark and the slits were then covered with Parafilm M. Three days after inoculation, 10 μl of sterile distilled water (SDW), bacteriocin preparations at different concentrations (2,000, 6,000, and 12,000 AU ml^{-1}), or Kocide solution (0.3%) was added to the wounds and the wounds were covered again with Parafilm M. Bacteriocin preparations were also added to uninoculated wounds as negative controls. After 7 days, the Parafilm M was removed. The experiment was carried out in duplicate on two different plants and was performed three times. (ii) For the second inoculation method, 10 μl of bacterial suspension containing 10^8 CFU ml^{-1} was applied to three scars obtained by detaching leaves. Leaf scars were then covered with Parafilm M for 3 days. Ten microliters of SDW, bacteriocin preparations (2,000 and 6,000 AU ml^{-1}), or Kocide solution (0.3%) was then applied to the scars, and the scars were covered again for 7 days with Parafilm M. Negative controls were obtained as described for the previous method. The experiment was carried out in duplicate with two different plants and was performed three times. For both inoculation methods, the plants were observed for symptom development for up to 60 days after inoculation. In order to quantify the reduction of symptom expression, overgrowths were excised from stems after 30 days and their weights were compared.

Epiphytic survival. The surfaces of twigs and leaves were cleaned with a paper towel moistened with 70% ethanol. Bacterial suspensions containing 10^8 CFU of strain PVBa229 or PVBa304 ml^{-1} and 0.03% of a wetting agent (Adiplant N; Bayer) were sprayed until runoff occurred. During spraying, a polystyrene shield was placed around each treated area to prevent drift to neighboring stems. One day after inoculation, SDW, cb (6,000 AU ml^{-1}), or Kocide solution (0.3%) was sprayed until runoff occurred. The number of bacteria on leaves and twigs was periodically evaluated (at 0, 2, 9, 15, and 30 days). Fifteen leaves and 20 cm of twigs were randomly collected at each sampling and separately added to 5 ml of an 0.85% NaCl aqueous solution in 50-ml tubes, which were vigorously shaken at room temperature for 2 h. The suspension was plated on KB agar containing cycloheximide (Sigma; 90 mg $liter^{-1}$) and incubated at 26°C for 3 days. The experiment was carried out in duplicate with two different plants and was performed three times. *P. syringae* subsp. *savastanoi* colonies were identified for counting purposes by colony morphology and typical blue fluorescence on KB agar. The identification was confirmed by growing 20% of representative colonies in KB agar and by evaluating indoleacetic acid production with the Salkowski assay technique (16).

Statistics. Data were analyzed by one-way analysis of variance followed by Dunnet's or Tukey-Kramer's multiple-comparison test. A P value of <0.05 was accepted as indicating statistical significance.

RESULTS

In vitro assays. The effect of bacteriocin preparations on growing cells of *P. syringae* subsp. *savastanoi* PVBa229 and PVBa304, compared with the effect of the commercial bactericide Kocide, is illustrated in Fig. 1. cs, cb, or fIV from *P. syringae* pv. ciccaronei added to growing cells of the two strains caused significant reductions in the numbers of viable cells after different periods of incubation. Analysis of variance followed by Dunnet's test for strain PVBa229 showed a significant difference ($P < 0.01$) in bacterial number with respect to the control after 2 h of treatment with Kocide, cb (6,000 AU ml^{-1}), or fIV (6,000 and 1,200 AU ml^{-1}) and after 8 h for all treatments. Twenty-four hours of incubation resulted in reductions of about 10^5, 10^7, and 10^9 CFU ml^{-1}, respectively, at concentrations of 100, 1,200, and 6,000 AU of bacteriocin preparations ml^{-1}, with minor differences between purification levels (Fig. 1). The effect of bacteriocin preparations on strain

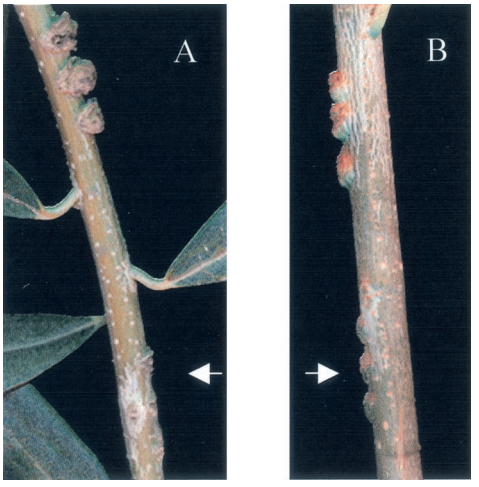

FIG. 2. Knot formation at V-shaped wounds 30 days after inoculation with *P. syringae* subsp. *savastanoi* PVBa229 (A) and PVBa304 (B). Arrows indicate sites treated with cb (6,000 AU ml^{-1}).

PVBa304 was similar to that on strain PVBa229, although PVBa304 was generally less sensitive (data not shown).

In planta experiments. Bacteriocin preparations affected symptom expression in olive stems inoculated with the pathogen. Thirty days after the inoculation of strains PVBa229 and PVBa304, typical knots appeared on the surfaces of inoculated sites with weights ranging from about 40 to 80 mg on V-shaped slits and from 20 to 50 mg on leaf scars. When bacteriocin preparations were added to the inoculated wounds, formation of smaller knots was observed. In particular, the application of 6,000 AU of cb ml^{-1} resulted in percentages of inhibition of 81.5% ± 4.9% (mean ± standard error) in V-shaped slits inoculated with strain PVBa229 (Fig. 2A) and 55.6% ± 1.2% in those inoculated with strain PVBa304 (Fig. 2B). In Fig. 3, the effect of the bacteriocin preparations is expressed as the percentage of knot inhibition obtained for inoculated sites treated with bacteriocin preparations with respect to that for untreated inoculated wounds. In the case of V-shaped wounds, for strain PVBa229, treatment with 2,000 AU of cs, cb, and fIV ml^{-1} resulted in percentages of inhibition of 9.3% ± 0.6%, 10.0% ± 2.1%, and 15.6% ± 2.3%, respectively (Fig. 3A). When more-concentrated preparations of bacteriocin were applied, the percentages of inhibition were 63.7% ± 2.9% (cs), 80.8% ± 4.9% (cb), and 64.3% ± 1.6% (fIV) (at a concentration of 6,000 AU ml^{-1}) and 79.0% ± 0.5% (cs), 75.2% ± 3.0% (cb), and 84.9% ± 2.1% (fIV) (at a concentration of 12,000 AU ml^{-1}) (Fig. 3A). For the leaf scar method, the inhibition values for the lowest concentration (2,000 AU ml^{-1}) were 29.8% ± 4.6% (cs), 52.4% ± 8.7% (cb), and 59.6% ± 3.9% (fIV), while for 6,000 AU ml^{-1}, they were 43.3% ± 1.2% (cs), 60.8% ± 3.8% (cb), and 79.9% ± 10.0% (fIV) (Fig. 3B). One-way analysis of variance of the data from the experiments on V-shaped slits (Tukey-Kramer's multiple-comparison test) showed a lower percentage of inhibition at a bacteriocin preparation concentration of 2,000 AU ml^{-1} with respect to that at the other concentrations and purification levels used (for both, $P < 0.001$). At higher concentrations, significant differences ($P < 0.01$) were obtained between purification levels for both cs and fIV. No difference ($P > 0.05$) was found between the inhibitory effects of cb at 6,000 AU ml^{-1} and all purification levels at 12,000 AU ml^{-1} (Fig. 3A). With the leaf scar method, the inhibitory effects of fIV at 2,000 and 6,000 AU ml^{-1} were not significantly different ($P > 0.05$) from the effects of cb at the same concentrations (Fig. 3B). Statistical analysis of strain PVBa304 showed the same behavior, although the percentages of inhibition were relatively lower. In particular, mean percentages of inhibition (± standard errors) for V-shaped wounds were 16.6% ± 3.9%, 50% ± 4.9%, and 60.8% ± 4.5% for treatments with 2,000 AU ml^{-1}, 6,000 AU ml^{-1}, and 12,000 AU ml^{-1}, respectively. With the leaf scar method, the mean percentages of inhibition (± standard errors) at 2,000 and

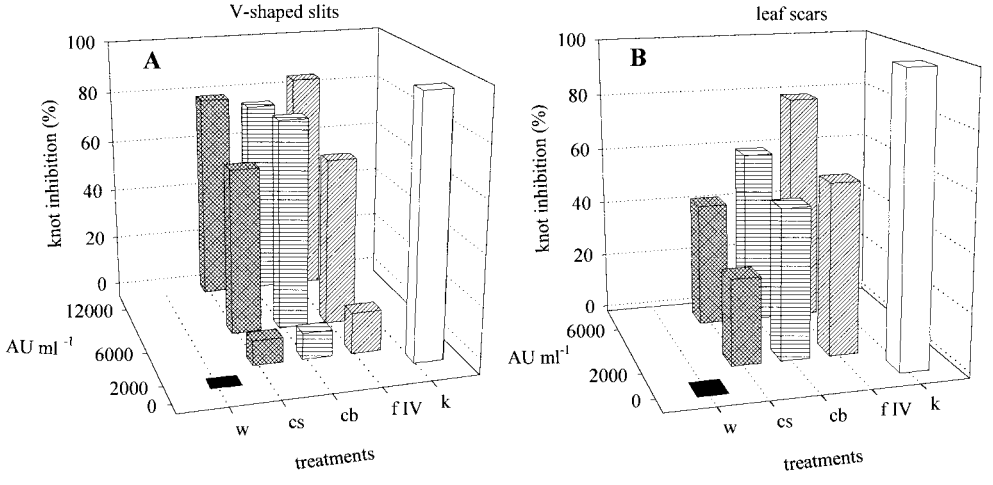

FIG. 3. Effects of *P. syringae* pv. ciccaronei NCPPB2355 bacteriocin preparations (cs, cb, and fIV) at different concentrations (2,000, 6,000, and 12,000 AU ml^{-1}), Kocide 101 (k) (0.3%), and SDW (w) on V-shaped slits (A) and leaf scars (B) of olive plants 30 days after inoculation with *P. syringae* subsp. *savastanoi* strain PVBa229. The effect was expressed as the percentage of inhibition of knot weight obtained for inoculated sites treated with bacteriocin preparations or Kocide 101 with respect to the knot weight of untreated inoculated wounds. Percentages of inhibition are for three independent experiments with two replicates each. Standard errors, calculated at a confidence level of 95% ($n = 18$), are reported in Results.

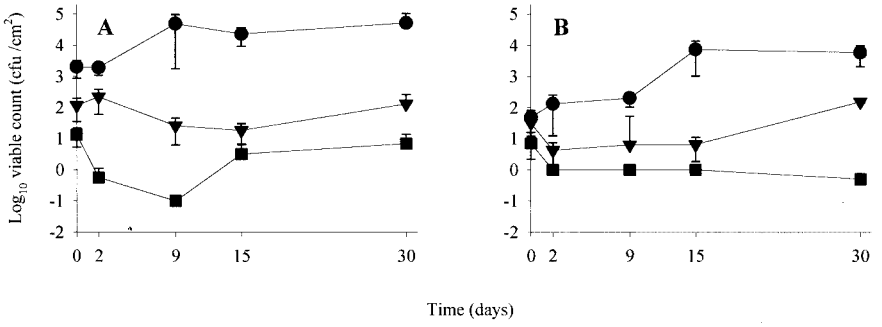

FIG. 4. Inhibition of the epiphytic survival of *P. syringae* subsp. *savastanoi* strain PVBa229 (●) on twigs (A) and leaves (B) of olive plants treated with *P. syringae* pv. ciccaronei NCPPB2355 cb (6,000 AU ml^{-1}) (▼) and Kocide 101 (0.3%) (■). Data, expressed as means ± standard errors (calculated at a confidence level of 95%), are from three independent experiments with two replicates each ($n = 6$).

6,000 AU ml^{-1} were 35.2% ± 2.6% and 58.2% ± 4.3%, respectively. Treatment with Kocide (0.3%) always resulted in almost complete inhibition of knot formation (Fig. 3). Uninoculated wounds treated with SDW or bacteriocin preparations appeared symptomless until the end of the observation period, and tissues healed about 15 days after inoculation. Analysis of variance of the percentages of inhibition did not show significant differences ($P > 0.05$) from experiment to experiment with strains PVBa229 and PVBa304 except for PVBa229 in leaf scar experiments in the case of cb and fIV at 6,000 AU ml^{-1} ($P > 0.048$ and 0.037, respectively).

Epiphytic survival. The epiphytic survival of *P. syringae* subsp. *savastanoi* strains was determined to be more relevant on twigs than on leaves at inoculation time, with recoveries of about 2.0×10^3 and 4.9×10 CFU/cm^2, respectively, for strain PVBa229 (Fig. 4) and of 3.0×10^2 and 1.2×10^2 CFU/cm^2, respectively, for the second strain used. The bacteria survived and multiplied until the end of our observations, reaching after 30 days a population of about 5×10^4 CFU/cm^2 on twigs and 6×10^3 CFU/cm^2 on leaves in the case of strain PVBa229 (Fig. 4). Treatment with the cb preparation (6,000 AU ml^{-1}) caused a reduction in bacterial multiplication on both twigs and leaves. In particular, on twigs, the populations recovered after 30 days were 400 (PVBa229) and 350 (PVBa304) times lower than the control populations, and on leaves, they were 40 (PVBa229) and 20 (PVBa304) times lower than the control populations. Treatment with a 0.3% solution of Kocide 101 caused the almost complete reduction of the epiphytic populations on both twigs and leaves (Fig. 4). Analysis of variance (followed by Dunnet's test) comparing results for PVBa229 control cells showed significant differences ($P < 0.01$) for Kocide 101 (0.3%) at 2, 9, 15, and 30 days and for 6,000 AU of cb ml^{-1} at 9, 15, and 30 days for both leaves and twigs. Similar results were obtained for PVBa304.

DISCUSSION

P. syringae subsp. *savastanoi* incited the formation of knots after inoculation on olive plants at a concentration of 10^5 or 10^8 CFU ml^{-1}. Treatments with bacteriocin from *P. syringae* pv. ciccaronei inhibited the formation of overgrowths caused by the pathogen. This effect reflects the inhibition of bacterial multiplication of the pathogen observed in in vitro experiments (Fig. 1). The inoculation methods (via V-shaped slits and leaf scars) were chosen to mimic damage caused by weather injuries or agronomic practices, since wounds allow epiphytic populations to invade and colonize host tissues. In the latter method, more-concentrated bacterial suspensions were used because leaf scars constitute narrow inoculation sites compared to V-shaped slits. In addition, only the two lowest bacteriocin concentrations were used, since in V-shaped inoculations, they were sufficient to reduce knot formation. The data obtained from experiments both in vitro and on olive plants led us to conclude that crude preparations of the bacteriocin can be used to control *P. syringae* subsp. *savastanoi*. In addition, we determined that 6,000 AU ml^{-1} is a concentration suitable to obtain an effective inhibition of olive knot formation using both strains (Fig. 2). In fact, in the case of strain PVBa229, treatment of the inoculated sites, V-shaped slits or leaf scars, with 6,000 AU of cb ml^{-1} resulted in the formation of knots, with weights reduced by 81 and 61%, respectively, compared to that of the control (Fig. 3). With strain PVBa304, the weight reductions obtained were 51 and 65%, respectively, using the same concentration of cb. The use of 12,000 AU ml^{-1} did not result in a proportional increase of knot inhibition. On the other hand, the amount of cb (7,000,000 AU) obtained by the purification of 1 liter of culture by a simple purification step (ammonium sulfate precipitation and subsequent dialysis) is enough to treat about 30 young olive plants (2 years old). The effects of bacteriocin on the behavior of both strains were similar, although PVBa304 showed a generally lower sensitivity in all experiments. To study the effect of the bacteriocin on the survival of an epiphytic population of the pathogen, 6,000 AU of cb ml^{-1} was used. This concentration was shown to be effective in controlling the multiplication of the pathogen. In fact, the inhibitory effect of only one treatment persisted until the end of observation since no regrowth was observed, while control cells continued to flourish (Fig. 4). The efficacy of the treatment was higher for twigs than for leaves probably because the rough bark surface offers suitable niches for pathogen survival and for the adhesion of treatment substances.

The reduction in the epiphytic survival of *P. syringae* subsp. *savastanoi* that was obtained by treating olive phylloplane with bacteriocin from *P. syringae* pv. ciccaronei is of great significance with respect to its effective use as a biological control agent. The epiphytic population size of bacterial plant pathogens is related to the probability of the disease, and control

methods should be able to eliminate resident populations or to prevent bacterial multiplication (11).

The use of bacterial metabolites instead of microbial agents should be recommended for overcoming difficulties that can be found in the application of microorganisms as biocontrol agents. In fact, the scarcity of information on strains and their ecology and the difficulty in obtaining the regulatory approval that is required for their formulation and application may represent disadvantages in the use of microorganisms in biological control (2). Another limitation may be the inability of some nonpathogenic bacteria to colonize protected sites and to compete with pathogenic bacteria (22). In our case, the addition of live *P. syringae* pv. ciccaronei cells to V-shaped slits 3 days after inoculation with strain *P. syringae* subsp. *savastanoi* PVBa229 did not result in the inhibition of knot formation (data not shown). This may be due to the incapacity of *P. syringae* pv. ciccaronei to multiply on olive tissues and to produce effective amounts of bacteriocin, as shown in previous experiments carried out in our laboratory (unpublished data).

In addition, the high specificity of action of bacteriocin from *P. syringae* pv. ciccaronei will result in selective alteration of the ecology of microorganisms present on the olive phylloplane. This is relevant to olive plants because of the presence on the phylloplane of useful microorganisms, such as lactic acid bacteria, that play an important technological role in olive fermentation processes (9). In particular, *P. syringae* pv. ciccaronei cb, tested in an agar spot assay, did not affect the growth of selected lactic acid bacteria isolated from olive phylloplane (data not shown).

The overall findings reported in this study lead us to conclude that bacteriocin from *P. syringae* pv. ciccaronei has the potential to control the survival of the causal agent of olive knot disease and to prevent its multiplication at inoculation sites. Further studies on the application of this bacteriocin to young nursery-grown olive plants are required to assess the number of treatments needed to obtain effective disease control and to ascertain the persistence of the bactericide.

Finally, these findings prompt new interest in bacteriocins produced by plant pathogens that can be considered an alternative biocontrol system useful in reducing the hazard associated with the use of synthetic pesticides. Valid formulations to ensure adequate effectiveness of the bactericide under natural environmental conditions should be pursued.

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